REMARKS

The Office Action dated July 5, 2005 has been carefully reviewed and the foregoing amendments are made in response thereto. In view of these amendments and the following remarks, Applicants respectfully request reconsideration and reexamination of this application and the timely allowance of pending claims.

I. Status of the Claims

Claims 1-18 and 23 remain pending and under examination.

II. Rejections under 35 U.S.C. §103(a)

Claims 1-10, 12, 14-18 and 23 were rejected under 35 U.S.C. §103(a) as allegedly being obvious over Huang (U.S. Patent No. 5,516,637) in view of Clark-Curtiss (Methods in Enzymology (1983) 101:347-362). The Examiner maintained the argument from the Office Action dated January 14, 2005 that one of ordinary skill in the art would be motivated to modify the teaching of Huang so as to have used bacterial minicells in place of bacterial cells to express a fusion protein, because the ordinary artisan would have allegedly recognized based on the disclosure of Clark-Curtiss that fusion proteins in minicells would have been more stable and more easily detected than in bacterial cells. Applicant respectfully maintains that there is no suggestion to combine the teachings of Clark-Curtiss and Huang, and therefore, the Examiner has failed to prove a *prima facia* case of obviousness. Applicant maintains the Clark-Curtiss reference teaches away from combining the references. In addition, Applicant respectfully asserts that the long felt need for a cell surface display system that does not have the limitations

of phage cell display and bacterial display systems is evidence that the Clark-Curtiss and Huang references would not motivate one of ordinary skill in the art to combine their teachings.

Applicant respectfully maintains the position that Clark-Curtiss does not teach that protein expression in minicells is more stable than in bacterial cells. Accordingly, one of ordinary skill in the art would not be motivated to combine the teachings of Clark-Curtiss and Huang as asserted in the Office Action. While the Examiner is correct to note that Clark-Curtiss teaches that minicells are useful for studying protein stability (see, *e.g.*, page 347, paragraph 3), Clark-Curtiss does not teach that protein stability is better in minicells. In fact, it would make no sense to use minicells as a test system to study protein stability if the stability were not comparable to wild-type cells. Clark-Curtiss does suggest that minicells with the *lon* mutation may be useful for expressing unstable proteins, but this disclosure pertains to the *lon* mutation and not to minicells in general. As support, Applicants have included with this response an article by C. Chung and A. Goldberg (*Proc. Natl. Acad. Sci. USA* 78: 4931-4935, 1981) which describes how the *lon* mutation in *E. coli* is associated with decreased capacity for proteolysis (page 4931, column 2, lines 3-5) and that the *lon* gene product is a protease (page 4935, column 1, lines 50-52). Applicants have also included with this response an article by S. Mizusawa and S. Gottesman (*Proc. Natl. Acad. Sci. USA* 80: 358-362, 1983) which states that *E. coli lon* mutants "have decreased ability to degrade abnormal proteins" (page 358, column 1, lines 1-3). These articles support Applicant's argument that it is the *lon* mutation, not the physiology of minicells, which confers the protein stability as discussed in Clark-Curtiss. Accordingly, Clark-Curtiss does not provide any evidence for the Examiner's blanket argument that protein expression is more stable in minicells than bacterial whole cells as providing motivation to combine the cited references.

Furthermore, the Examiner's statement that the Applicant's present claims encompass the use of any type of minicell, including the minicells of Clark-Curtiss having a *lon* mutation, is misplaced. The fact remains that one of ordinary skill in the art would not be motivated to combine the teachings of Clark-Curtiss with the teachings of Huang, as discussed above, because Clark-Curtiss does not provide that a minicell, independent of mutations responsible for decreased capacity to degrade proteins, confers greater protein stability than a bacterial cell. Rather, it is the *lon* mutation which confers greater protein stability in both *E. coli* and *E. coli* minicells because such mutants have decreased capacity to degrade protein as previously discussed.

The Examiner also argued that there is no evidence in Clark-Curtiss that the stability of minicells is comparable to whole cells and cited from Clark-Curtiss that "Many proteins specified by 'foreign' DNA in *E. coli* are unstable, being subject to degradation by various proteolytic enzymes. Plasmids containing minicells can be conveniently used to investigate the occurrence, rate, and extent of such instability" (page 361). However, this passage is proposing the use of minicells in general to study protein stability, not to improve protein stability. Again, there is no teaching in this passage or in the whole Clark-Curtiss reference that proposes that proteins are generally more stable in minicells.

The Examiner has also maintained that it would be obvious to combine the teachings of Clark-Curtiss and Huang because "the ordinary artisan would have recognized that synthesis of the fusion protein in minicells...would have enhanced the specificity of detection of the fusion protein because minicells are not subject to a high background of chromosomal gene products which occurs when using bacterial cells" (page 4, lines 1-6 of Office Action). However, the Examiner has failed to provide any evidence from either reference suggesting an advantage to

using minicells for displaying fusion proteins on the cell surface that is related to the lack of chromosomal DNA in minicells. "The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination." *In re Millis*, 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990).

Further, Applicant maintains the position that Clark-Curtiss teaches away from the claimed invention because the reference teaches "that not all protein-synthesizing systems function in minicells and that some systems function less well in minicells than in vegetative cells" and that it has been hypothesized that this "may be due to nuclease or ribonuclease activity in minicells or to competition between promotor sequences for limiting amounts of RNA polymerase present in minicells" (page 359, lines 36 to page 360, line 2). The Examiner has disagreed on the basis that "obviousness does not require absolute predictability but only the reasonable expectation of success." Nevertheless, the Federal Circuit has made it clear that a prior art reference must be considered in its entirety, including disclosures that teach away from the claims. *W.L. Gore & Asso., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983); see also MPEP § 2141.02.

The Examiner has argued that the statement made in Clark-Curtiss (described above) is not a "general teaching away" because Clark-Curtiss also teaches that plasmid-containing minicells have been useful for studies on the replication, recombination, and repair of plasmid DNA and for studies on the synthesis of plasmid-specified gene products, the stability of macromolecules, the study of protein processing mechanisms, and the localization or excretion of plasmid-specified proteins. However, these passages relate to studying how a cell processes proteins or DNA and are clearly not relevant to the methods of displaying fusion proteins on the cell surface, as claimed in the present invention. Therefore, the cited passages do not justify

ignoring the disclosure in Clark-Curtiss which teaches away from using minicells to express proteins for a screening method.

Finally, Applicant also asserts that the long felt need for an effective and rapid method for generating and screening large libraries of peptides such as described in the present application is further evidence of its lack of obviousness. *See Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 1538, 218 USPQ 871, 879 (Fed. Cir. 1983) ("evidence rising out of the so-called 'secondary considerations' must always when present be considered en route to a determination of obviousness"); *see also* MPEP 716.01(a). Indeed, while both bacterial display systems and minicells have been used for twenty years (page 1 of instant specification and Clark-Curtiss *et al.* reference), no one had put the two together to achieve a more effective and robust screening method.

For instance, the present specification describes how fusion proteins comprising pilin protein (TraT) or flagellar protein (FliC) and thioredoxin (the FLITRX™ display system) had been used in bacterial display systems to overcome problems associated with localizing fusion proteins on the bacterial cell surface (page 3, lines 2 through 19). Despite these advancements, there were additional limitations to these bacterial display systems which significantly restricted the ability to use these systems to effectively generate and screen large libraries. For example, there are significant peptide size restraints for these systems. At the time of filing, the largest peptides to be displayed by fimbriae ranged 50 to 60 amino acids and the largest peptides fused to FliC flagellin of *E. coli* appeared to be 302 amino acids (page 4, lines 8-13). In addition, these systems are unable to accommodate amino acid analogs *in vivo* and require cumbersome protocols to accommodate amino acid analogs *in vitro* (page 4, lines 17-19). Furthermore,

peptides which are toxic to the bacterial cell and therefore lethal cannot be screened using the display systems known in the art at the time of filing (page 5, lines 2-4).

Minicell display, on the other hand, addresses the above limitations and thereby satisfies a long felt need for an effective and rapid method for generating and screening large libraries of peptides. This long felt need is further evidenced by the licensing of the technology to Sopherion Therapeutics. *See In re Tiffin*, 443 F.2d 344, 170 USPQ 88 (CCPA 1971) (fact that affidavit supporting contention of fulfillment of a long-felt need was sworn by a licensee adds weight, as long as there is a *bona fide* licensing agreement entered into at arm's length).

For the above-described reasons, Applicant respectfully submits that the Examiner has not established the burden required for a *prima facie* case of obviousness. Reconsideration and withdrawal of the rejection under 35 U.S.C. §103(a) based on the Clark-Curtiss and Huang references are respectfully requested.

Claim 11 was rejected under 35 U.S.C. §103(a) as being unpatentable over Huang in view of Clark-Curtiss and further in view of Shivakumar *et al.* (Plasmid. 1979. 2:279-289). Applicants respectfully traverse this rejection. As previously discussed, one of ordinary skill in the art would not be motivated to combine the teachings of Huang relating to expression of fusion proteins in bacterial cells with the teachings of Clark-Curtiss relating to minicells, because Clark-Curtiss teaches away from using minicells for protein expression and is further evidenced by the long felt need for an effective cell surface display tool. The Shivakumar *et al.* reference does not cure the deficiencies of Huang and Clark-Curtiss. Accordingly, reconsideration and withdrawal of the §103 rejection based on the combination of Huang, Clark-Curtiss and Shivakumar are respectfully requested.

Claims 4 and 13 were rejected under 35 U.S.C. §103(a) as being unpatentable over Huang in view of Clark-Curtiss and further in view of Georgiou (U.S. Patent No. 5,348,867). Applicants respectfully traverse this rejection. As previously discussed, one of ordinary skill in the art would not be motivated to combine the teachings of Huang relating to expression of fusion proteins in bacterial cells with the teachings of Clark-Curtiss relating to minicells, because Clark-Curtiss teaches away from using minicells for protein expression and is further evidenced by the long felt need for an effective cell surface display tool. The Georgiou reference does not cure the deficiencies of Huang and Clark-Curtiss. Accordingly, reconsideration and withdrawal of the §103 rejection based on the combination of Huang, Clark-Curtiss and Georgiou are respectfully requested.

In view of the arguments submitted above, Applicants respectfully request reconsideration and withdrawal of the 35 U.S.C. §103 rejections.

Conclusion

This reply is fully responsive to the Office Action dated July 5, 2005. Therefore, a Notice of Allowance is next in order and is respectfully requested.

Except for issue fees payable under 37 CFR 1.18, the commissioner is hereby authorized by this paper to charge any additional fees during the pendency of this application including fees due under 37 CFR 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-1283. This paragraph is intended to be a **CONSTRUCTIVE PETITION FOR EXTENSION OF TIME** in accordance with 37 CFR 1.136(a)(3).

If the Examiner has any further questions relating to this Reply or to the application in general, she is respectfully requested to contact the undersigned by telephone so that allowance of the present application may be expedited.

Dated: January 5, 2006

Respectfully submitted,
COOLEY GODWARD LLP

COOLEY GODWARD LLP
Customer No. 58249
875 15th Street, NW, Ste. 800
Washington, DC 20005
Tel: 202-842-7800

By: _____
Heather Boussios
Reg. No. 52,704

Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 358–362, January 1983
Biochemistry

Protein degradation in *Escherichia coli*: The *lon* gene controls the stability of sulA protein

(cell division/SOS regulation/bacteriophage λ)

SAEKO MIZUSAWA* AND SUSAN GOTTESMAN†

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Communicated by Ira Pastan, September 30, 1982

ABSTRACT *Escherichia coli lon* mutants are defective in the ATP-dependent proteolysis of abnormal proteins. The mutants are also sensitive to ultraviolet light (UV) in that septation is inhibited after exposure to UV. *sulA* mutations, isolated as suppressors of UV sensitivity unlinked to *lon*, do not affect proteolysis but allow septation to occur after DNA damage. We have confirmed the hypothesis that the product of the *sulA* gene is degraded by *lon* proteolysis. If sulA (the product of *sulA*) is a UV-inducible division inhibitor, as suggested by a variety of experiments, lon (the product of *lon*) may regulate cell division by regulating the half-life of sulA. We cloned the *sulA* gene in a bacteriophage λ vector from a plasmid carrying the *ompA* region of *E. coli*. An 18-kilodalton polypeptide was identified as the product of the *sulA* gene. Pulse–chase labeling demonstrated that the half-life of the sulA protein is 1.2 min in *lon*$^+$ cells and 19 min in *lon*$^-$ cells. This work demonstrates that lon proteolysis affects the stability of a native *E. coli* protein.

The *lon* mutations of *Escherichia coli* affect a variety of physiological processes. *lon* mutants have decreased ability to degrade abnormal proteins (1–4), overproduce capsular polysaccharide (5), and are defective for lysogeny of bacteriophages λ and P1 (6, 7). They are also sensitive to DNA-damaging agents, such as ultraviolet light (UV) or methyl methanesulfonate (MeMes) (8). This UV sensitivity seems to result from an exaggeration of the normal inhibition of *E. coli* cell division after DNA damage (9). *lon* mutants do not recover from this division arrest and are therefore UV and MeMes sensitive.

Mutations identified in *lon* strains as suppressors of sensitivity to UV or MeMes map at two loci, *sulA* and *sulB*. These suppressors block the lethal filamentation seen in *lon* strains but do not affect other *lon* phenotypes (10–14). *sulA* and *sulB* also do not affect the cell's UV repair system or its induction. George and her co-workers (15) proposed that a division inhibitor, induced after UV irradiation, might be a target for the *lon* protease. The properties of *sulA* mutations suggest that the *sulA* gene product may be an inhibitor of septation, induced after DNA damage (14, 16). *sulB* mutations, which are rare relative to *sulA*, are dominant and may be in a gene encoding an essential cell division function that is a target for the *sulA* inhibitor. If sulA, the product of the *sulA* gene, were unstable and inactivated by lon proteolysis, its persistence in *lon* mutants might lead to excessive filamentation (13–15).

Recently the product of the *lon* gene has been identified as an ATP-dependent protease (17, 18), suggesting that the primary defect of the *lon* mutants may be the defect in proteolysis. If so, *lon* must exert its pleiotropic effects on cellular physiology by affecting the half-life of proteins involved in such processes as capsular polysaccharide synthesis or septation inhibition. Thus far, however, the lon protease has only been demonstrated to affect the degradation of abnormal proteins and the bacteriophage λ N protein *in vivo* (19). *In vitro*, it digests artificial substrates such as casein and hemoglobin (18). In the work described here we have asked directly if a protein implicated in the control of one set of *lon*-perturbed functions is a target for the lon protease.

We have cloned the *sulA* gene, identified the gene product, and examined the stability of sulA in *lon*$^+$ and *lon*$^-$ strains. We show evidence that sulA is degraded by lon proteolysis. Our results support the hypothesis of George *et al.* (15) and are sufficient to explain the UV sensitivity of *lon* strains.

MATERIALS AND METHODS

Bacteria and Phage Strains. The important bacterial and phage strains used are listed in Table 1. All strains not listed are from the National Institutes of Health strain collection. The procedure for P1 transduction has been described (14). Tetracycline-sensitive derivatives (Tet^s) of strains carrying transposon Tn*10* were isolated by the procedure of Maloy and Nunn (22). λ phages λimm21*c*I and λimmλ*c*Ib2 were used as *int*$^+$ helper phages to lysogenize λSM1 and λSM5, respectively, at the bacterial attachment site. The presence of the *sulA* and *ompA* mutations was determined by resistance to MeMes (3) and K3 phage (23), respectively.

Preparation of DNA. DNA of plasmid pTU100 was prepared according to the procedure of Clewell and Helinski (24). Closed circular DNA molecules were purified by a CsCl/ethidium bromide equilibrium density gradient centrifugation followed by dialysis against 10 mM Tris·HCl, pH 7.5/1 mM EDTA. High-titer phage stocks were prepared by the method of Yamamoto *et al.* (25). The phage were concentrated by polyethylene glycol precipitation and further concentrated and purified by CsCl equilibrium density gradient centrifugation. The purified phage were dialyzed against 10 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$. Phage DNA was extracted with phenol and dialyzed against 10 mM Tris·HCl, pH 7.5/1 mM EDTA.

Procedure for UV-Inducible Filamentation. UV treatment and preparation of samples for microscopy were carried out as described (14). Samples were fixed and kept in 2% (wt/vol) formaldehyde solution, collected, and resuspended in 10 mM $MgSO_4$ before layering on slides and were observed by phase-contrast microscopy in a Zeiss instrument.

***N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine Mutagenesis of *sulA* Transducing Phage.** An *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-mutagenized lysate was prepared essentially by following the procedure of Adelberg *et al.* (26). N99 (F$^-$ *gal rpsL*) was

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Abbreviations: MeMes, methyl methanesulfonate; Tet^s, tetracycline sensitive; kb, kilobase(s); kDal, kilodalton(s).

* Present address: The Institute of Medical Science, The University of Tokyo, 4-6-1, Shirokanedai Minato-ku Tokyo 108, Japan.
† To whom reprint requests should be addressed.

Table 1. Bacterial and bacteriophage strains

Strain name	Genotype	Source
HU100(pTU100)	F⁻ *thi pyrD gltA galK trp recA ompA rpsL* (pTU100)	U. Henning (20)
SM8	F⁻ *his pyrD leu rpsL*	SG13107 (14); select Tet^s
SM32	F⁻ *his pyrD Δlon-100 gal rpsL*	P1 (*galE*::Tn*10*) to SG13009 (14); Tet^s
SM37	F⁻ *his leu Δlon-100 sulA366 gal rpsL*	P1 (*galE*::Tn*10*) to SG13082 (14); Tet^s
SM39	F⁻ *his pyrD sulB367 Δlon-100 gal rpsL*	P1 (*galE*::Tn*10*) to SG13083 (14); Tet^s
SM40	F⁻ *his leu Δlon-100 sulA366 gal trp*::Tn*10 supF*	P1 (*trp*::Tn*10 supF*) to SM37
λD69	*Bam1° srlΔ*(1, 2) *imm*21 *nin*5 *Hind6°*	(21)
λSM1	*ompA⁺ sulA⁺ Bam1° srlΔ*(1, 2) *int imm*21 *nin*5 *Hind6°*	This work; orientation 1
λSM2	Same as for λSM1; orientation 2	This work
λSM4	*Bam1° srlΔ*(1, 2) cI857 *nin*5	λD69 × λ*bio*11 *nin*5 cI857
λSM5	*ompA⁺ sulA⁺ Bam1° srlΔ*(1, 2) *int* cI857 *nin*5	λSM1 × λ*bio*11 *nin*5 cI857

grown to 2×10^8 cells per ml in TBMM medium (10 g of Bactotryptone per liter/86 mM NaCl, 10 mM $MgCl_2$/0.2% maltose) at 37°C, infected with λSM5 at a multiplicity of infection of 0.5, and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (10 μg/ml) for 15 min at 37°C. Mutagen-treated infected cells were collected and resuspended in 2 vol of prewarmed L broth (15 g of tryptone, 5 g of NaCl, and 5 g of yeast extract per liter), distributed in four aliquots, and incubated for 90 min at 37°C. The resultant lysate was treated with $CHCl_3$ and centrifuged to remove debris. Under these conditions, the frequency of clear mutations was 1–2% and the viability of the noninfected cells was 30–50%.

Protein Synthesis in UV-Irradiated Bacteria. The procedure was modified from that of Jaskunas *et al.* (27). Unless otherwise indicated, the cells were grown and treated in minimal medium M56 (28) supplemented with 0.2% maltose, uracil at 15 μg/ml, and 18 amino acids (excluding methionine) each at 25 μg/ml. At an OD_{650} of 0.3, the cells were concentrated 5-fold in medium supplemented with histidine and leucine (required amino acids, both at 25 μg/ml) instead of the 18 amino acids, irradiated with an incident dose of 1,000 J/m^2 at 254 nm, diluted 1:5 in the medium containing the 18 amino acids, and incubated at 37°C for 10 min in the dark. The cells were collected, resuspended in the medium containing 10 mM $MgCl_2$ at a concentration of 2×10^9 cells per ml, infected with phage at a multiplicity of infection of 5, and left on ice for 20 min to allow phage to adsorb. The cells were incubated in a 37°C water bath for 2 min, then diluted 1:20 with prewarmed medium and incubated at 37°C for an additional 5 min. Labeling of the proteins was started by adding [^{35}S]methionine (20 μCi/ml, 1,000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) to the samples and stopped after 5-min incubation by adding ¼ vol of 50% trichloroacetic acid/0.5% unlabeled methionine. In some cases UV-treated cells were labeled with a ^{14}C-labeled amino acid mixture (New England Nuclear, 10 μCi/ml, 55 Ci/mol of carbon) in medium supplemented with required amino acids. The labeled cells were collected by centrifugation, washed with cold acetone, dried, and suspended in 40 μl of sample buffer for NaDodSO$_4$/polyacrylamide gel electrophoresis. The gel electrophoresis was carried out by the procedure of King and Laemmli (29) except that the gels were 7.5–20% linear gradient gels. In most cases gels were fixed in 50% trichloroacetic acid for 30 min and then soaked in 25% methanol/10% acetic acid (vol/vol) for at least 1 hr. Some gels were stained in 50% trichloroacetic acid/2.5% Coomassie brilliant blue for 30 min and destained in 25% methanol/10% acetic acid. The labeled protein bands were visualized by fluorography (30). To estimate the molecular weight of the labeled proteins, the following proteins were used as standards: phosphorylase b (subunit molecular weight, 94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), ferritin (18,500), and α-lactalbumin (14,400).

For quantitative data, a portion of the gel corresponding to the protein band of interest was cut out and soaked in 0.1 ml of distilled water at room temperature for 2 hr. Then 0.3 ml of Protosol and 4 ml of Econofluor (both from New England Nuclear) were added, and the mixture was incubated in a 37°C water bath overnight before radioactivity was measured.

RESULTS

Cloning of the *sulA* Gene. To allow cloning of the *sulA* gene, we have looked for closely linked markers to use for selection. The *sulA* gene cotransduces with *pyrD* at a frequency of 50–65% (3, 10, 13); *ompA*, coding for one of the major outer membrane proteins of *E. coli*, has a similar cotransduction pattern, and lies on the same side of *pyrD* as *sulA* (10). In P1 transduction experiments, *sulA* and *ompA* were very closely linked to each other (data not shown).

The *ompA* gene has been cloned in low-copy-number plasmids (20, 31). A 1.78-kilobase (kb) *Bam*HI fragment that carries the sequence for the amino terminus of the *ompA* protein and is sufficient to encode ompA complementing activity was taken from the low-copy-number plasmid pTU100 and cloned in λD69 by inserting into the *Bam*HI site in the λ *int* gene (Fig. 1). Phages were isolated with the *Bam*HI fragment in either orientation; both phages λSM1 and λSM2 complemented *ompA*.

A λ*sulA⁺* phage should restore MeMes sensitivity to a *lon sulA* host. λSM1 lysogens of the *lon sulA* strain SM37 were isolated, with the prophage located either at *attB* or near the *ompA* locus. λSM2 lysogenizes poorly in the *lon sulA* strain, although lysogens can be isolated in a *proC lon⁺ sulA* strain and the *lon* mutation subsequently can be introduced by P1 transduction, selecting for proline independence and screening for mucoidy. We believe the difficulty in establishing lysogens is due to overproduction of sulA (see *Discussion*). Both λSM1 and λSM2 lysogens of *lon sulA* were sensitive to MeMes, and λSM2 lysogens were slightly filamentous even in the absence of MeMes. λ carrying the 1.78-kb fragment also complement *sulA* for UV-inducible filamentation; a *lon sulA*(λ*sulA⁺*) lysogen forms long filaments after UV treatment, as does a *lon sul⁺* strain. This effect is specific to *sulA*; the transducing phage does not cause filamentation in *lon⁺* or *lon sulB* strains after UV treatment. These results indicate that the 1.78-kb fragment carried by λ*sulA⁺* contains the *sulA* gene and that this gene is functional in a lysogen.

Isolation of Phages Carrying *sulA* Mutations. On *lon* bacterial lawns, plaques of an *imm*λcI857 derivative of λSM1, λSM5*sulA⁺*, are clear, whereas they are turbid on *lon⁺* lawns. The simplest explanation for this phenomenon is that multiple

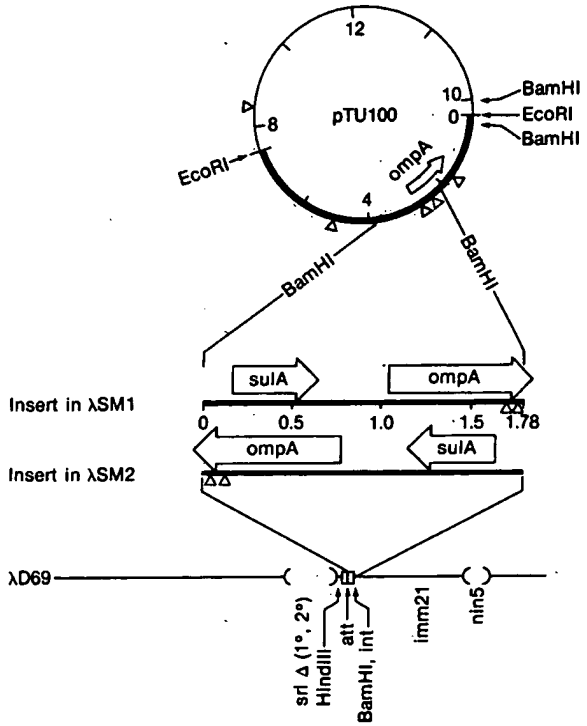

FIG. 1. Construction of λSM1 and λSM2. A 1.78-kb *Bam*HI fragment of pTU100 was inserted into the *Bam*HI site of the λD69 vector phage (21) and packaged *in vitro* according to the procedure of Enquist and Sternberg (32). Phages with inserts in the *int* gene were detected by the red plaque test (33). The orientation of the insert was determined by digestion with *Hin*dIII and *Pvu* II (the sites are indicated as triangles). The restriction map of pTU100 and schematic diagram of the reading frames for the ompA and sulA proteins are cited from Henning *et al.* (20) and Beck and Bremer (34), respectively. In the Beck and Bremer sequence of this region, sulA protein is indicated as a hypothetical 17-kilodalton (kDal) protein.

infection with sulA$^+$ transducing phage leads to enough sulA expression to kill lysogens growing in the plaque. Consistent with this explanation is the observation that in a lon sulB strain, in which sulA may not be able to act, λSM5 plaques are not as clear as on *lon* or *lon sulA* strains. To isolate a *sulA* mutation on the λSM5 phage, the *lon sulA* strain SM37 was lysogenized with λSM5. From this MeMes-sensitive lysogen, MeMes-resistant colonies were selected. Phage induced from the MeMes-resistant lysogens no longer complement *sulA*, still complement *ompA*, and form turbid plaques on *lon* hosts.

The difference in plaque morphology for phage carrying *sulA*$^+$ and *sulA*$^-$ was used to screen for additional *sulA* mutants on the phage. λSM5 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and 50 turbid-plaque-forming phage were isolated from 2,800 plaques on a *lon* bacterial lawn. Thirty-five of these were *sulA* mutants as judged by their failure to complement *sulA* for MeMes sensitivity. One mutant, λ*sulA46*, acts as if it has an amber mutation: lysogens of a *lon sulA supF* strain form filaments on MeMes agar, whereas lysogens of a *sup*$^+$ strain do not.

Twelve λ*sulA* mutants, including *sulA46*, were shown to still be *ompA*$^+$. Therefore, *ompA* and *sulA* act as separate genes.

Identification of the Gene Product. The proteins coded for by the 1.78-kb fragment carried by λSM5 were determined by infection of UV-irradiated cells followed by labeling with [^{35}S]methionine (Fig. 2) or [^{14}C]leucine (data not shown). Compared with infection with the λ parental vector, three additional protein bands with the molecular masses of 27, 24, and 18 kDal were observed after infection with λSM5. The 18-kDal protein is apparently the product of the *sulA* gene. The 18-kDal protein band is missing after infection with 4 of 11 mutant phages, including *sulA46* (Fig. 2*A*), whereas the 27- and 24-kDal proteins are present after infection with all 11 mutants. λSM5*sulA46*, carrying an amber mutation in *sulA*, produced the 18-kDal protein in *supF* host cells (Fig. 2*B*). The 27- and 24-kDal protein bands are consistent with the size for a truncated ompA protein (24 kDal) and its unprocessed precursor (pro-ompA, 27 kDal) (34).

Stability of the sulA Protein. Is the sulA protein degraded by a *lon*$^+$-dependent process? The stability of the sulA protein was assayed in *lon*$^+$ and *lon*$^-$ strains by pulse–chase labeling of the proteins synthesized after infection of UV-irradiated hosts with the *sulA* transducing phage. Host cells were treated with UV, infected with λSM5, pulse labeled with [^{35}S]methionine, and chased with an excess of unlabeled methionine. Samples

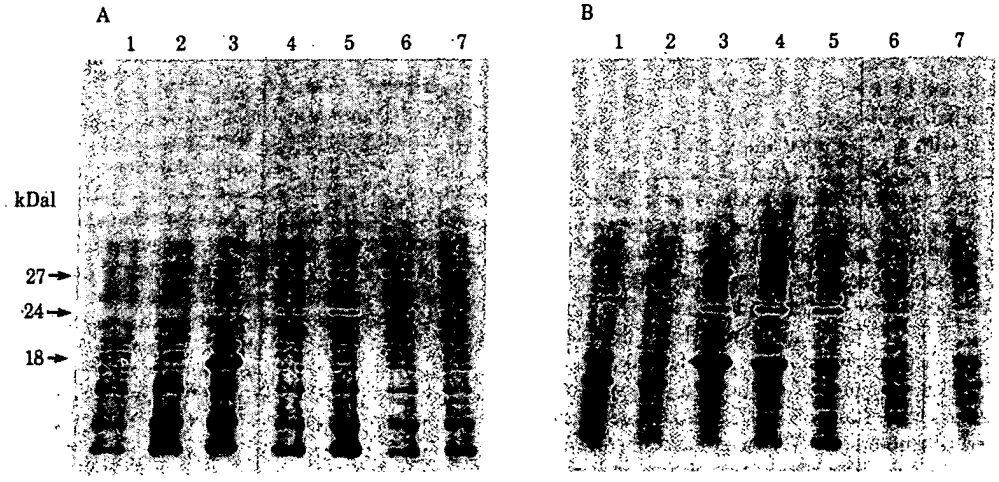

FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis of proteins synthesized after infection of UV-irradiated cells. Cells were *sup*$^+$ (SM37) in *A* and *supF* (SM40) in *B*, both lysogenic for λ*ind*. Cells were infected after UV irradiation as follows: lane 1, no infection; lane 2, vector, λSM4; lane 3, wild-type phage, λSM5*sulA*$^+$; lane 4, λSM5*sulA46*; lane 5, λSM5*sulA41*; lane 6, λSM5*sulA24*; lane 7, λSM5*sulA11*. The arrows indicate the position of the proteins specified by the 1.78-kb insert carried by λSM5*sulA*$^+$.

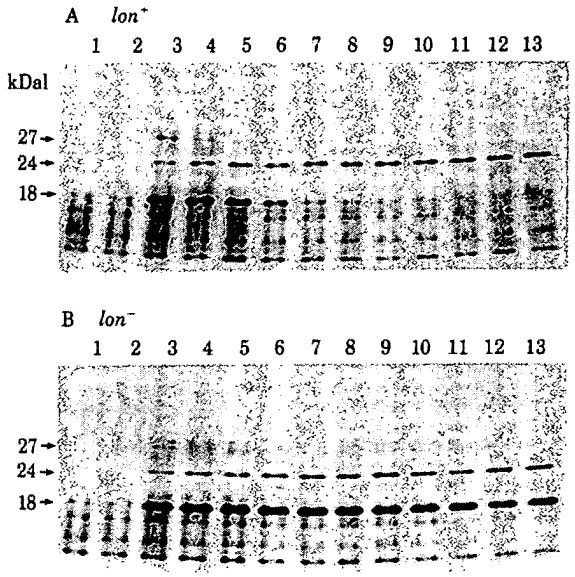

FIG. 3. Pulse-chase labeling of sulA in *lon*$^+$ and *lon*$^-$ cells. Cells were UV irradiated, and infected with λSM5, as for Fig. 2. Infected cells were pulse labeled with [^{35}S]methionine for 1 min and chased with 10^4-fold excess unlabeled methionine. Cells were *lon*$^+$ (SM8) in *A*, *lon*$^-$ (SM32) in *B*; both are lysogenic for *λind*. Controls are lane 1, no infection and, lane 2, infection with vector phage, λSM4, and sampled at 0 min of chase. Lanes 3–13, cells were labeled for 1 min (lane 3) and chased for 1 min (lane 4), 2 min (lane 5), 3 min (lane 6), 5 min (lane 7), 7.5 min (lane 8), 10 min (lane 9), 12.5 min (lane 10), 15 min (lane 11), 17.5 min (lane 12), and 20 min (lane 13).

were removed at various times after the pulse labeling and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. As shown in Figs. 3 and 4, the sulA protein has a half-life of 1.2 min in *lon*$^+$ cells and 19 min in *lon*$^-$ cells. Among the *E. coli* proteins coded for by λSM5, lon protease is specific for sulA; no decay

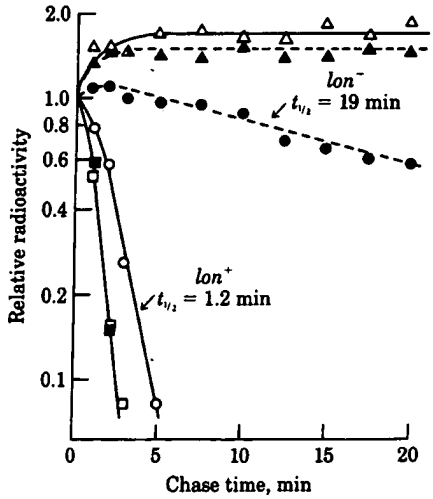

FIG. 4. Stability of sulA in *lon*$^+$ and *lon*$^-$ cells. The pulse–chase-labeled bands of the sulA, ompA, and pro-ompA proteins were cut out from the gels shown in Fig. 3, and the radioactivity in the bands was measured. The radioactivity remaining in the band is presented as relative to that found at 0 min of chase. Open symbols, bands from infection of *lon*$^+$ strain; closed symbols, bands from infection of *lon*$^-$ strain. △ and ▲, 24-kDal (ompA) band; □ and ■, 27-kDal (pro-ompA); ○ and ●, 18-kDal (sulA) band.

of the ompA protein was detected in either *lon*$^+$ or *lon*$^-$ cells. The half-life of the pro-ompA protein, 0.3 min, was the same in *lon*$^+$ and *lon*$^-$ cells. Similar experiments were carried out in a *lon sulB* strain; the *sulB* mutation has no effect on the stability of the sulA protein (data not shown).

DISCUSSION

Cell division in *E. coli* is normally coupled to DNA replication, so that anucleate cells are rare. DNA damage leads to inhibition of septation while cell mass increases, resulting in formation of nonseptated filaments. As DNA damage is repaired, normal cell division resumes. In *lon* cells, however, an exaggerated and lethal continuation of filamentation occurs (9).

sulA has been implicated in this process because mutations in *lon* cells that block lethal filamentation map in *sulA*, or, less commonly, in *sulB*. No other aspect of the SOS induced DNA repair system is apparently affected by the *sulA* and *sulB* mutations. George and her co-workers (15) postulated that a septation inhibitor coded for by one of the *sul* genes might be induced in response to DNA damage and that its persistence might be regulated by *lon* through proteolysis. The recent finding that *sulA* is transcriptionally induced after DNA damage (16) makes sulA the best candidate for such a division inhibitor.

We have cloned the *sulA* gene on a 1.78-kb *Bam*HI fragment, in a λ vector. Lysogens of λ carrying the fragment in either orientation complement *sulA*, suggesting that the fragment codes for the entire *sulA* gene, including its promoter. An amber mutation in *sulA*, *sulA46*, allows identification of the gene product as a protein with a molecular mass of 17–18 kDal. The fragment also encodes the amino terminus of the ompA protein, but our mutant analysis indicates that the two proteins are coded for by separate genes. Beck and Bremer (34), studying *ompA*, determined the DNA sequence of the whole *Bam*HI fragment and noted an open translational reading frame that could encode an 18-kDal protein. Because the sizes of the sulA protein and the hypothetical protein are identical, and no other open reading frame found on the fragment could encode a polypeptide with molecular weight near this, we propose that the open reading frame codes for the sulA protein.

The class of DNA-damage-inducible genes shares the property of being under control by *lexA* and *recA* as part of the SOS system (16, 35). For a number of these genes, a *lexA* binding site has been found in the promoter region (36–38). Upstream of the open reading frame for the *sulA* gene there are three potential promoter sequences. In addition, we have found a region in the Beck and Bremer sequence (at base pairs 130–149) highly homologous with the *lexA* binding site regions identified in the *recA*, *lexA*, and *uvrB* genes. Our potential *lexA* binding site shares 18/20 bases of homology with the lexA binding site of the *recA* gene. This region overlaps the −10 regions of two of the three promoters. The finding of this site is consistent with the finding by Huisman and D'Ari (16) that *sulA–lacZ* operon fusions act genetically as if they are repressed by *lexA*.

Huisman and D'Ari also found, in their *sulA–lacZ* fusion experiments, that *lon* does not transcriptionally regulate *sulA*. We found that *lon* regulates activity of *sulA* by degradation of the sulA protein. We have demonstrated that sulA is unstable in *lon*$^+$ cells, disappearing with a half-life of 1.2 min. In *lon*$^-$ cells, however, the half-life is 19 min. Our data are sufficient to explain the filamentation phenotype of *lon* mutants: if sulA is a division inhibitor and is synthesized for a short time after DNA damage, its persistence in *lon* cells would lead to extended inhibition of septation and therefore lethal filamentation. In *lon*$^+$ cells, the rapid decay of sulA would ensure a return to normal septation as soon as new sulA synthesis is shut off.

If sulA product is the only inducible function necessary for inhibition of septation, any mechanism that leads to its persistence would be sufficient for filamentation. Lysogens of λSM2 carry *sulA* downstream from the λ promoters p_L and p_{int}, in the proper orientation to be expressed from p_{int}. These strains filament slightly even in the absence of DNA-damage-inducing treatment in *lon* strains. Thus if p_{int} is expressing *sulA* at a low constitutive level (39), it may be sufficient to cause filamentation of *lon* host cells without any necessary induction of other SOS functions.

Thus we have demonstrated that lon degrades native *E. coli* proteins, as well as nonsense fragments (3) and a variety of denatured proteins (18). Among a number of unstable λ proteins examined, only the positive regulatory protein N was stabilized by lon (19). The half-lives of neither the pro-ompA protein nor the stable ompA protein are affected by the *lon* system. We conclude that lon is not generally responsible for the processing of membrane proteins. This is in contrast to the suggestion of Gayda *et al.* (40), who have proposed that the processing of the precursor of an outer membrane protein is regulated by lon. The other phenotypes of *lon* such as the overproduction of capsular polysaccharide in *lon* cells have not yet been satisfactorily explained. If the mechanism of *lon* control of this phenomenon is similar to its control of cell division, we would predict that a positive regulator of polysaccharide synthesis should exist, and its stability would be regulated by lon proteolysis.

The recA protease has an even more striking specificity than lon, cleaving only a handful of phage repressors and the cellular repressor lexA. The half-life of the λ control protein cII may be regulated by the cellular *hf1* function (41). Therefore, *E. coli* may utilize a set of proteases for fine-tuning and timing regulation of important cellular processes.

We thank U. Henning for sending us pTU100, T. Silhavy for K3 phage and *ompA* mutant strain K3R6, and Doug Ward for the λD69 cloning vector. We are grateful to Max Gottesman, Don Court, Michael Gottesman, Nancy Craig, and Sankar Adhya for discussions and for their comments on the manuscript, to Ray Steinberg for photography, and to Annette Kuo for her help in preparing the manuscript.

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Proc. Natl. Acad. Sci. USA
Vol. 78, No. 8, pp. 4931–4935, August 1981
Biochemistry

The product of the *lon* (*capR*) gene in *Escherichia coli* is the ATP-dependent protease, protease La

(proteolytic enzymes/energy requirement/intracellular protein degradation)

CHIN HA CHUNG AND ALFRED L. GOLDBERG

Department of Physiology, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115

Communicated by Bernard D. Davis, May 21, 1981

ABSTRACT In *Escherichia coli*, degradation of abnormal proteins is an energy-requiring process; it is decreased in mutants in the *lon* (*capR* or *deg*) gene. We find that the protein encoded by the *lon* gene is an ATP-dependent protease and is identical to protease La, recently described in *E. coli*. Both proteins are serine proteases that hydrolyze casein and globin, but not insulin, in the presence of ATP and Mg^{2+}. Both respond to ATP, less well to other nucleoside triphosphates, and not to nonhydrolyzable ATP analogs. The purified *lon* protein has an apparent M_r of 450,000 and appears to be composed of four identical subunits. Its size, chromatographic behavior, and sensitivity to various inhibitors and heat are indistinguishable from those of protease La. Moreover, in a strain that carries additional copies of the lon^+ allele on a plasmid, the content of protease La, but not of other proteases, is 2- to 10-fold greater than in the lon^+ parent strain. Strains carrying the nonsense mutations *capR9* and $capR^-$ also contain this ATP-dependent proteolytic activity, but it is present in substantially lower amounts and is inactivated by phosphocellulose chromatography, unlike the wild-type enzyme. Degradation of abnormal proteins in these lon^- strains, which is slower than in the wild type, still requires ATP. Alterations in the ATP-dependent protease in the lon^- mutants can account for the defect in intracellular proteolysis and perhaps also for the other phenotypic effects of this pleiotropic gene.

Like eukaryotic cells, *Escherichia coli* has an efficient system for selectively degrading abnormal polypeptides (1, 2), such as may arise by nonsense (3–5) or missense mutations (6) or by incorporation of puromycin or amino acid analogs (7). One of the intriguing features of the breakdown of abnormal proteins (and also of normal proteins) in bacterial and mammalian cells is that this process requires metabolic energy (1, 8–11). Studies with metabolic inhibitors have demonstrated that ATP is required for an initial endoproteolytic step in the degradation of abnormal proteins in *E. coli* (5); in extracts, degradation of globin and of nonsense fragments of β-galactosidase can be stimulated 2- to 4-fold by ATP (12, 13). Swamy and Goldberg (10, 11, 14, 15) have demonstrated that *E. coli* contains eight distinct proteases, and one of these, named "protease La," is completely dependent on ATP and Mg^{2+} for activity. Recently, Larimore *et al.* (16) have shown that proteolysis by this enzyme requires concomitant hydrolysis of the ATP. Various observations further suggest that, *in vivo*, protease La catalyzes the rate-limiting, ATP-dependent steps for protein breakdown (14, 16).

Mutants with decreased ability to hydrolyze abnormal proteins have been isolated by Bukhari and Zipser (4) and were initially called *deg*. These mutations were located at 11.4 min on the *E. coli* genetic map and were shown to coincide with *lon* (also called *capR*) mutations (4, 16). All such mutations result in many other phenotypic alterations, including overproduction of capsular polysaccharides due to derepression of biosynthetic enzymes, defective cell division leading to filament formation, and increased sensitivity to UV light and to radiomimetic agents (18–22). It is not clear which of these effects is primary and how the decreased capacity for proteolysis is related to the other phenotypic effects of the *lon* mutation.

In vivo, the lon^- mutation was shown to cause a decrease in the same initial cleavages of nonsense polypeptides that are prevented by ATP depletion (5). The identification of a single ATP-dependent protease in *E. coli* (10, 11, 14, 15) raised the possibility that this enzyme might be the product of the *lon* gene or might be regulated by it. One argument against this possibility is that extracts of lon^- strains showed some ATP-stimulated proteolysis (12). Recently, the *lon* gene product was purified as a DNA-binding protein by Zehnbauer *et al.* (23) and shown to be a 94,000-dalton polypeptide by $NaDodSO_4$/polyacrylamide gel electrophoresis.

The present studies were therefore undertaken to test whether the purified *lon* gene product might be a protease, to clarify its possible relationship to protease La, and to examine the effects of lon^- mutations on this activity.

MATERIALS AND METHODS

The following *E. coli* K-12 strains were provided for these investigations by A. Markovitz: RGC121 which carries the wild-type lon^+ ($capR^+$) allele, RGC121/pJMC40 which carries the lon^+ ($capR^+$) allele both on the chromosome and on plasmid pSC101, RGC123 which is isogeneic with RGC121 but contains the lon^- (*capR9*) allele, and another lon^- ($capR^-$) strain (24). The *capR9* and $capR^-$ are both nonsense mutations and have identical lon^- phenotypes, but the *capR9* allele (unlike the $capR^-$) is dominant to the wild type when present on a plasmid (23).

Bacteria were grown to stationary phase in Luria broth at 37°C. For the strain carrying plasmid pJMC40, the medium was supplemented with tetracycline at 5 μg/ml (24) to prevent loss of the plasmid. For phosphocellulose chromatography (23), one-half of the harvested cells were washed, resuspended, and lysed in buffer P [0.1 M K phosphate, pH 6.5/10 mM 2-mercaptoethanol, 1 mM EDTA, 20% (vol/vol) glycerol]. Chromatography on phosphocellulose was carried out by the method of Zehnbauer *et al.* (23). For analysis on DEAE-cellulose columns the other half was suspended in buffer D (10 mM Tris·HCl, pH 7.8/5 mM $MgCl_2$). Preparation of cell-free extracts and chromatography on DEAE-cellulose were performed as described (14).

The purified lon^+ ($capR^+$) and lon^- (*capR9*) gene products were provided for these studies by M. F. Charette and A. Markovitz. These polypeptides had been purified as DNA-binding proteins and identified as 94,000-dalton polypeptides (23), which we confirmed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Partially purified protease La was kindly provided by F. S. Larimore. It was prepared as described by Larimore *et al.* (16) from frozen *E. coli* K-12 (Grain Processing, Muscatine, IA).

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α,β-Methylene-ATP, β,γ-methylene-ATP, and adenosine 5'-(O-3'-thiotriphosphate) were obtained from PL Biochemicals; DEAE-cellulose (DE-52) and phosphocellulose (P-11) were purchased from Whatman. ^{125}I-Labeled insulin (^{125}I-insulin) was obtained from Cambridge Nuclear Corp. [^{3}H]Methylcasein and [^{14}C]methylapohemoglobin were prepared as described (25).

Proteolytic activity against these substrates was assayed as described by Swamy and Goldberg (14). All assays were carried out in 25 mM Tris·HCl, pH 7.8/5 mM $MgCl_2$ in the presence and absence of 1 mM ATP. The incubations were for 0.5–1 hr at 37°C with either the purified *lon* gene product or partially purified protease La (16) in a final volume of 0.5 ml. The reaction was stopped by the addition of 60 μl of 100% (wt/vol) trichloroacetic acid and 40 μl of bovine serum albumin (30 mg/ml) as a carrier. The assay tubes were kept on ice for 30 min and, after centrifugation, the amount of acid-soluble radioactivity in 0.4 ml of the supernatant was assayed in 4 ml of Liquiscint (National Diagnostics, Parsippany, NJ). The acid-soluble products of ^{125}I-insulin hydrolysis were assayed in a Beckman gamma spectrometer.

The degradation of puromycin-containing polypeptides *in vivo* was measured as described by Goldberg (7). The various strains were grown at 37°C in M9 medium supplemented with 0.2% glucose, thiamine at 5 μg/ml, and leucine, tryptophan, proline, and adenine at 50 μg/ml.

RESULTS

In the presence of ATP and Mg^{2+} the *lon* gene product that had been purified as a DNA-binding protein hydrolyzed [^{3}H]casein and [^{14}C]globin to acid-soluble material but did not digest ^{125}I-insulin (Table 1, Exp. A). In a typical experiment using casein at 30 μg/ml and 5 mM $MgCl_2$, half-maximal proteolytic activity (K_m) was observed with 7.8 μM ATP. By contrast, no activity was evident with ATP or Mg^{2+} alone (data not shown). Because casein was hydrolyzed more rapidly, all further experiments were performed with this substrate. The pattern of substrates cleaved (Table 1, Exp. A) and the requirement for ATP and Mg^{2+} resemble earlier observations with the partially purified protease La (14, 16). The preparation of the *lon* protein used in these studies was approximately 90% pure 94,000-dalton polypeptide as analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (data not shown). Identical proteolytic activity was observed when the *lon* gene product was purified to homogeneity by an adaptation (15) of the procedure of Zehnbauer *et al.* (23) (unpublished observations).

Table 1. Proteolytic activity of the purified *lon* (*capR*) gene products

Substrate	μg substrate hydrolyzed/hr/mg enzyme: No ATP	With ATP
Exp. A: With *capR*$^+$ protein		
[^{3}H]Casein	2.2	712.0
[^{14}C]Globin	0.8	73.9
^{125}I-Insulin	0.9	1.1
Exp. B: With *capR9* protein		
[^{3}H]Casein	0.1	0.0
[^{14}C]Globin	0.0	0.0

The *capR*$^+$ protein, which was purified to near homogeneity, and the *capR9* gene product, which was partially purified on phosphocellulose, were both provided by M. F. Charette. Both proteins appeared to be 94,000-dalton polypeptides.

Table 2. Effects of different nucleotides and ATP analogs on casein hydrolysis by the *lon* gene product

Nucleotide or analog (1 mM)	Activity, %
ATP	100
ADP	3
AMP	0
GTP	14
UTP	65
CTP	74
Adenosine 5'-(O-3'-thiotriphosphate)	29
α,β-Methylene-ATP	18
β,γ-Methylene-ATP	0

Similar data were previously obtained with protease La by Larimore *et al.* (16).

Comparisons of Protease La and *lon* Gene Product. Larimore *et al.* (16) have recently shown that protease La requires cleavage of ATP for its proteolytic activity. Proteolysis was inhibited by sodium vanadate, a potent ATPase inhibitor (26), and the effect of ATP could not be reproduced with the nonmetabolizable ATP analog β,γ-methylene ATP or with the poorly metabolizable analogs α,β-methylene-ATP and adenosine 5'-(O-3'-thiotriphosphate). CTP and UTP stimulated protease La activity (16) but less effectively than ATP, and other nucleotides tested had little or no effect. Nearly identical results with these nucleotides and analogs were obtained for the *lon* gene product (Table 2), which thus also seems to require ATP cleavage for proteolytic activity.

Because of the similarities between the two ATP-dependent proteolytic activities, they were further compared with respect to sensitivity to various inhibitors, stability against heat, and molecular weight. Like protease La (16), the *lon* gene product was sensitive to diisopropyl fluorophosphate and therefore probably is a serine protease. N-Ethylmaleimide and antipain also inhibited the two activities to a similar extent (Table 3).

Larimore *et al.* found that protease La is rapidly inactivated at 42°C but that ATP could prevent this thermal inactivation (16). In the absence of ATP the activity of the *lon* gene protease decreased by about 80% upon preincubation at 42°C for 1 hr but, when ATP was added, no loss in activity occurred (Table 3).

Upon glycerol density gradient centrifugation, the *lon* gene

Table 3. Effects of inhibitors and temperature on the ATP-dependent proteolytic activity of the *lon* gene product and protease La

Additions	Activity, % Protease La	*lon* protein
Exp. A		
Control	100	100
Diisopropyl fluorophosphate:		
1 mM	65	70
10 mM	5	9
N-Ethylmaleimide (5 mM)	35	37
Antipain (30 μg/ml)	66	70
Sodium vanadate (0.1 mM)	25	11
Exp. B		
Preincubation for 1 hr at 42°C:		
No ATP	21	14
With ATP	100	100

These data compare the purified *lon*$^+$(*capR*$^+$) gene product (23) and partially purified protease La (16). Neither preparation showed any proteolytic activity in the absence of ATP. The data on heat stability of protease La were taken from Larimore *et al.* (16).

product and protease La showed identical molecular weights of 450,000 (Fig. 1). This value was confirmed by gel filtration on a Sephacryl S-300 column from which both activities were eluted as sharp peaks (data not shown; see Table 4). Zehnbauer *et al.* (23) found that the *lon* gene product, purified as a DNA-binding protein, migrated as a 94,000-dalton protein on Na-DodSO$_4$/polyacrylamide gel electrophoresis. Thus, the native *lon* gene product probably is composed of at least four, or at most five, identical 94,000-dalton subunits. Furthermore, our most purified preparations (15) of protease La contain a major band of 94,000 daltons as well as several contaminating polypeptides (data not shown). These similarities in size further argue that the *lon* gene product and protease La are identical.

Protease La Activity of *lon*$^+$ and *lon*$^-$ Strains. To obtain clearer evidence on whether or not the *lon* gene encoded protease La, the levels of this enzyme were compared in a wild-type strain, a wild-type strain carrying additional copies of the *lon*$^+$ allele on a plasmid, and *lon*$^-$ (*capR9* and *capR*$^-$) mutants. Cell-free extracts from these strains were prepared and fractionated by chromatography on DEAE-cellulose to separate protease La from the ATP-independent proteases (14, 15). The activity of the protease La peak was 2- to 10-fold greater (depending on the experiment) in the strain carrying additional copies of the *lon*$^+$ allele than in the wild-type parent (Fig. 2). Conversely, both *lon*$^-$ mutants showed some ATP-dependent proteolytic activity eluting where protease La is found but in significantly lower amounts (approximately 50%) than in the wild-type strain (Fig. 2; Table 4). Similar results were found in three additional experiments. In contrast, the levels of the various ATP-independent proteolytic activities against casein were indistinguishable in all four strains (Fig. 2). Thus, by all criteria tested, the *lon* gene product is identical to protease La.

To confirm that the ATP-dependent proteolytic activity remaining in these mutants corresponds to protease La, the ATP-stimulated proteins obtained from the DEAE-cellulose column (Fig. 2) were pooled and compared with the wild-type enzyme (Table 4). On gel filtration with Sephacryl S-300, the activity from *capR9* and *capR*$^-$ showed the same size (450,000 daltons) as the wild-type protein. In addition, the enzymes from these two mutants lost most of their activity when incubated at 42°C for 1 hr but both could be completely stabilized by addition of ATP, as had been observed with wild-type enzyme (15) and purified *lon* gene product (Table 3).

In our initial studies, we observed that the *capR9* gene product purified on phosphocellulose did not show any ATP-dependent proteolytic activity (Table 1, Exp. B), in contrast to the results obtained with DEAE-cellulose (Fig. 2). A possible explanation for this apparent contradiction is that purification by phosphocellulose chromatography may selectively inactivate the mutant protease. Therefore, cell-free extracts were prepared and fractionated on phosphocellulose as described by Zehnbauer *et al.* (23). The wild-type protease could be isolated from this column (15) with greatly increased specific activity (Fig. 3). However, no ATP-dependent proteolytic activity was recovered after phosphocellulose chromatography of the *capR9* and *capR*$^-$ strains. Zehnbauer *et al.* (24) have shown that the 94,000-dalton *capR9* polypeptide could be eluted from a phosphocellulose column in a similar fashion as the *capR*$^+$ gene product. Therefore, the ATP-dependent proteolytic activity from the mutants appears to be more labile than that from the

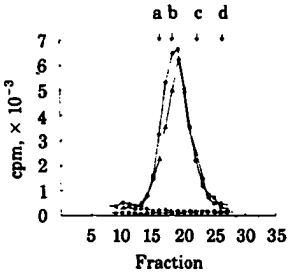

FIG. 1. Comparison of protease La and the *lon* gene product in glycerol density gradient centrifugation. The *lon* gene product (●, ○) and protease La (▲, △) preparations were applied onto 5 ml of 10–30% glycerol density gradients. These preparations contained 24 and 325 μg of protein, respectively. Centrifugation was then performed at 200,000 × *g* for 6 hr. Fractions (7 drops; 150 μl) were collected from the bottom and 50 μl of each fraction was assayed in the presence (●, ▲) and absence (○, △) of ATP. Arrows, peaks of marker proteins: a, thyroglobulin, 669,000; b, β-galactosidase, 467,000; c, catalase, 240,000; d, alkaline phosphatase, 82,000.

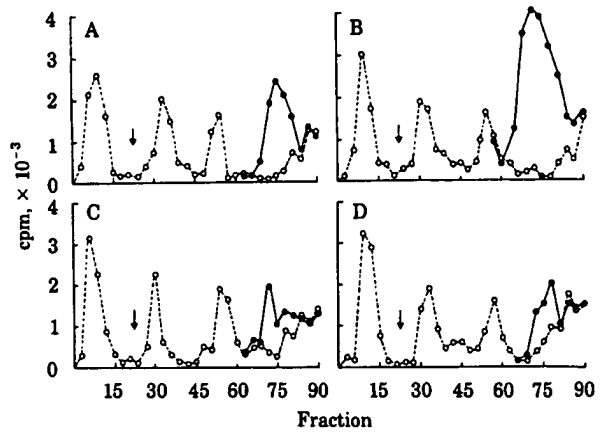

FIG. 2. Proteolytic activities in *E. coli* strains carrying different *lon* alleles fractionated by DEAE-cellulose chromatography. Cell-free extracts were prepared in buffer D from an *E. coli* strain carrying the *lon*$^+$ allele (*A*), a derivative carrying additional copies of the *lon*$^+$ allele on a plasmid (*B*), a derivative carrying a mutant *lon*$^-$ allele, *capR9* (*C*), and another *lon*$^-$ strain, *capR*$^-$ (*D*). After dialysis against the same buffer, the extracts (each containing 342 mg of protein) were adsorbed to DEAE-cellulose columns (1.5 × 18 cm) which were washed with two bed volumes of buffer D. The proteins were eluted with linear 0–0.25 M gradients of NaCl in buffer D in a total volume of 500 ml. Fractions (4 ml) were collected at a flow rate of 35 ml/hr and assayed against [^{3}H]casein in the presence (●) and absence (○) of ATP (1 mM). Arrows indicate the beginning of gradient. The peak of the proteolytic activity observed only in the presence of ATP eluted at the position of protease La (14–16). Proteolytic activity elsewhere in the gradient was not stimulated by ATP (14).

Table 4. Protease La activities in *lon*$^+$ and *lon*$^-$ strains

	capR$^+$	*capR9*	*capR*$^-$
Total activity:			
Recovered from DEAE-cellulose, %*	100	47	51
Size on Sephacryl S-300, daltons	450,000	450,000	450,000
Activity after 1 hr at 42°C:†			
No ATP, %	27	39	31
With ATP, %	100	100	100

The data on enzyme recoveries were obtained from DEAE-cellulose chromatography as in Fig. 2. These results are the averages of four independent experiments that gave similar results. The measurements on size and heat stability were performed twice on material from the DEAE-cellulose columns. In each case, a single symmetric peak of ATP-dependent proteolytic activity was observed. The column was 1.0 × 27 cm and was eluted with buffer D containing 10% glycerol.

* Relative to wild type.

† Relative to activity present initially.

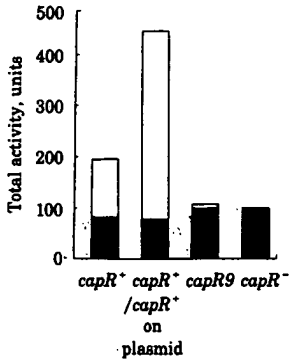

FIG. 3. Proteolytic activities in *E. coli* strains carrying different *lon* alleles, eluted from phosphocellulose columns. Cell-free extracts were prepared in buffer P from the same *E. coli* K-12 strains studied in Fig. 2. After dialysis against this buffer, the extracts (each containing 410 mg of protein) were adsorbed to phosphocellulose columns (1.5 × 18 cm) which were washed with two bed volumes of buffer P. The adsorbed proteins were eluted with buffer P containing 0.35 M phosphate. The nonadsorbed fractions did not contain ATP-stimulated proteolytic activity, but contained 20 times more ATP-independent activity (data not shown). The eluted proteins were assayed against [^{3}H]casein in the presence (open bar) and absence (solid bar) of ATP. Units are defined as μg of casein hydrolyzed per hour.

wild type. In related unpublished studies, we have found that the inactive *capR9* protein eluted from phosphocellulose is capable of inhibiting the wild-type protease, and this property may account for its phenotypic dominance (23, 24).

Energy Requirement for Protein Degradation in *lon*$^+$ and *lon*$^-$ Cells. These observations would predict that the *lon*$^-$ mutants *in vivo* still degrade proteins by an ATP-dependent process, but do so more slowly than the wild-type cells. We therefore compared the rates of degradation of puromycin-containing polypeptides in the *capR*$^+$, *capR9*, and *capR*$^-$ cells under normal conditions and when energy metabolism was inhibited. Both *capR9* and *capR*$^-$ mutations resulted in a 50% reduction in degradation of puromycyl polypeptides (Fig. 4) as previously observed with other *lon*$^-$ strain (27, 28). In both mutant and wild type cells, removal of glucose from the medium

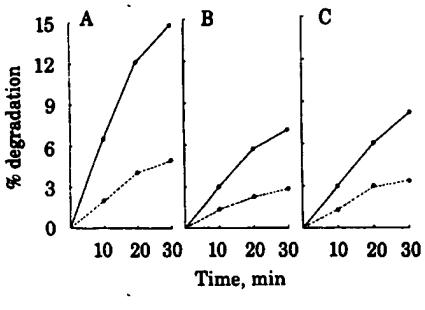

FIG. 4. Effects of energy depletion on the degradation of puromycin-containing polypeptides in *capR*$^+$ (*A*), *capR9* (*B*), and *capR*$^-$ (*C*) strains. To induce production of abnormal proteins (7), growing cultures of the *lon*$^+$, *lon*$^-$ (*capR9*), and *lon*$^-$ (*capR*$^-$) strains were incubated with puromycin at 100 μg/ml for 15 min. [^{14}C]Phenylalanine was then added for 5 min to label incomplete proteins synthesized under these conditions. These cultures were washed to remove unincorporated phenylalanine and the puromycin. One-half of the cells were resuspended in the growth medium (●) and the other half, in medium containing 0.1 mM 2,4-dinitrophenol and lacking glucose (○). The washes and incubation medium contained a large excess (300 μg/ml) of nonradioactive phenylalanine to prevent reincorporation of [^{14}C]phenylalanine. Aliquots were withdrawn at each time point, and acid-soluble radioactivity was measured.

and addition of the uncoupling agent 2,4-dinitrophenol decreased the degradation of abnormal protein by approximately 70%. Thus, in accord with the cell-free data (Fig. 2), an ATP-dependent proteolytic system functions in the strains carrying the *capR9* or *capR*$^-$ mutation, but its capacity to degrade abnormal proteins is decreased.

DISCUSSION

The *lon* gene product, purified as a DNA-binding protein, has been demonstrated to be an ATP-dependent protease; by all criteria tested, it is identical to protease La. The purified *lon* gene product and partially purified protease La are of similar size (Fig. 1), have similar specificities (i.e., hydrolyze casein and globin but not insulin) (Table 1; refs. 14–16), are eluted similarly from DEAE-cellulose columns (Fig. 1; ref. 23), are serine proteases as shown by their sensitivity to diisopropyl fluorophosphate, are equally sensitive to *N*-ethylmaleimide and vanadate (Table 3), and are rapidly inactivated at 42°C but stabilized by ATP (Table 3). Both proteins show proteolytic activity only in the presence of ATP and Mg^{2+} (probably an ATP^{2-}–Mg^{2+} complex) and they show similar responses with various nucleotides and with nucleotide analogs (Table 2; ref. 16). Finally, the strains carrying additional copies of the *lon*$^+$ gene on a plasmid show an increased level of protease La activity but not of the other *E. coli* proteases; nonsense mutations in this locus decrease this activity specifically (Fig. 2). We therefore refer to protease La and the *lon* gene product interchangeably.

It is of particular interest that cleavage of ATP appears to be essential for proteolytic activity. Casein hydrolysis is not stimulated by nonmetabolizable ATP analogs (Table 2; ref. 16) and is sensitive to vanadate, a potent inhibitor of various ATPases (Table 3; ref. 26). The enzymological mechanisms linking ATP cleavage and proteolytic activity are not clear. Related studies have demonstrated that this unique protease has ATPase activity which is stimulated by protein substrates (ref. 29; unpublished data). In addition, Larimore *et al.* (16) have shown that protease La does not have protein kinase, protein adenylase, or ubiquitin-conjugating activity (30, 31). The direct activation of protease La by ATP is not consistent with the model suggested by Hershko, Rose, and colleagues (30, 31) to account for the ATP requirement for intracellular proteolysis in reticulocytes. According to their model, ATP is required by a multiple-component system that conjugates a small polypeptide, ubiquitin, to protein substrates to enhance their susceptibility to ATP-independent proteases (30, 31).

The requirement for ATP hydrolysis also differentiates the *lon* gene product from the other known proteolytic enzymes, including those activated by ATP. During induction of prophage λ, in the presence of ATP the *recA* gene product catalyzes the endoproteolytic cleavage of the phage repressor (32). However, that reaction does not seem to require ATP hydrolysis because it occurs severalfold more effectively with adenosine 5′-(*O*-3′-thiotriphosphate) than with ATP, unlike that with the *lon* gene product. Proteases from liver or reticulocyte cytoplasm have been described that they are stimulated 2- to 3-fold by ATP, but they also do not require ATP hydrolysis (33, 34).

The ATP requirement of protease La can account for the energy requirement for degradation of normal and abnormal proteins *in vivo* (1, 8–11). Earlier studies showed that in the degradation of nonsense fragments of β-galactosidase the *lon* mutation affects the same endoproteolytic step that is decreased by inhibitors of energy metabolism (5). Furthermore, both the *capR9* and *capR*$^-$ mutations caused a similar decrease, of about 50%, in protease La activity (Fig. 2) and in the capacity of the cells to degrade puromycyl polypeptides (Fig. 4). In addition, the relatively high affinity of this enzyme for ATP can account for the finding that intracellular ATP levels must be decreased

to <10% of normal levels before protein degradation decreases (2, 9). These various observations support the earlier suggestion (14, 15) that protease La is responsible for the rate-limiting steps (5) in the degradation of abnormal proteins *in vivo*.

We have previously demonstrated ATP stimulation of proteolysis in crude extracts of *lon*$^-$ as well as *lon*$^+$ strains (12). In the present studies, protease La was demonstrated by DEAE-cellulose chromatography to be present in *lon*$^-$ extracts, but in reduced amounts (Fig. 2). The mutant enzyme was also shown to resemble the wild type in multimeric size and heat stability (Table 4). Furthermore, although decreased in rate, the degradation of abnormal proteins in these mutants cells still required energy (Fig. 4). In additional experiments, extracts from a strain (N5115) carrying a deletion within the *lon* gene (kindly provided by Susan Gottesman) were found to contain protease La activity in lower amounts than in the wild-type parent (N5116), in accord with the results of Fig. 2. In such cells, the degradation of abnormal proteins also occurred at a decreased rate and still required energy (unpublished data). The presence of the ATP-dependent protease even in the nonsense and the deletion mutants and our failure to find strains truly lacking this activity suggest that this enzyme may be essential for the survival of the cells.

Although phosphocellulose chromatography is particularly useful in purifying the ATP-dependent protease from the extracts of wild-type cells (15), this step completely destroyed the activity in the *capR9* and *capR*$^-$ extracts. This inactivation explains our initial failure (Table 1) to find the residual ATP-dependent proteolytic activity in *lon*$^-$ mutants as well as the conclusion of Charette *et al.* (29) that these mutants completely lack this activity.

Both *capR*$^-$ and *capR9* are nonsense mutations. The 50% reduction observed in the total activity of protease La in these mutants (Fig. 2) may underestimate the effect of the mutation on enzyme function because the *capR9* strain has been reported to overproduce this polypeptide (23, 24). The *capR9* mutation must be located close to the carboxyl terminus because its polypeptide, purified as a DNA-binding protein, is indistinguishable from that of the wild-type on $NaDodSO_4$/polyacrylamide gel electrophoresis (24) and on gel filtration on a Sephacryl S-300 column (Table 4). It is noteworthy that, after phosphocellulose chromatography has inactivated the *capR9* enzyme, the protein retains DNA-binding activity (23) and it can inhibit the wild-type protease La (unpublished data). The finding of an ATP-dependent proteolytic activity in the *capR*$^-$ extract is particularly interesting because Zehnbauer *et al.* (23) were unable to find the 94,000-dalton polypeptides in such extracts. However, we did observe a normal-size multimeric enzyme in this strain (Table 4).

The demonstration that the *lon* gene product is a protease provides a possible explanation for the ability of *lon* mutations to affect a remarkable variety of physiological processes. Among these are capsular polysaccharide synthesis, cell division, filamentation, transcription of the *gal* operon, and sensitivity to UV light and radiomimetic agents (18–22). Possibly these various processes involve short-lived proteins that are normally degraded by the ATP-dependent proteolytic enzyme. For example, *lon*$^-$ cells are defective in lysogenization of phage λ, and one of the short-lived regulatory proteins involved in this process, the *N* gene product, is stabilized by the *lon*$^-$ mutation (35). A similar decrease in the proteolytic inactivation of various cellular regulatory proteins or enzymes may account for many other phenotypic consequences of the *lon* mutation.

This work was initiated in this laboratory as a collaboration with Mr. M. F. Charette and Dr. A. Markovitz who preferred to publish independently (29). We are very grateful to Professors Howard Green and Bernard D. Davis for their helpful advice. Our colleagues Drs. Fred S. Larimore, Sreedhara Swamy, Lloyd Waxman, and Lian Yeh have made many helpful suggestions and provided materials used in this study. We also thank Ms. Maureen Rush for help in preparation of this manuscript. Our studies have been supported through grants from the National Institute of Neurological and Communicative Diseases and Stroke, the Juvenile Diabetes Foundation, and the Kroc Foundation.

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